**Original Research Article** **Pharmaceutical Science**

**International Journal of Pharma and Bio Sciences**

**ISSN 0975-6299**

# ALGINATE BASED NANOPARTICLES AS A CARRIER MATRIX FOR THE DELIVERY OF CALIXARENE DERIVATIVE AS PHARMACEUTICAL COMPOUND

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## ABSTRACT

Modern medicines are in urgent need of hyphenating new therapeutics, which can act as antimicrobial agent. Calixarenes are versatile macromolecules which can serve as a platform for the design and development of biologically active therapeutics. But the presence of aromatic ring structure in the calixarenes skeleton renders it hydrophobic which requires a hydrophilic carrier to reach the target cell efficiently for drug action. The present work is a comparative study of antimicrobial activity of sodium salt of p-sulfonato-calix-4-arene (calixarene derivative) in aqueous solution (phosphate buffer saline) and encapsulated in hydrophilic nanocarrier – alginate nanogel cross-linked with $Ca^{+2}$/$Ba^{+2}$. The alginate nanogels were characterized with respect to their size, encapsulation efficiency and their drug release profiles. Antimicrobial activity of p-sulfonato-calix-4-arene and alginate nanogels loaded with the p-sulfonato-calix-4-arene was determined by disc diffusion method and colony forming unit (CFU) method against drug resistant strain of *Escherichia coli* (MTCC 443). The experimental results suggest that the encapsulated calixarene derivative in hydrophilic calcium alginate nanogels is potentially best candidate against *E.coli* strain at much lower concentration than the free calixarene derivative soluble in phosphate buffer saline (PBS).

**KEYWORDS:** *Alginate nanogels; Drug Delivery; Hydrophilic carrier; Calixarene derivatives*

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**Received on: 25-03-2017**
**Revised and Accepted on: 20-06-2017**
**DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.3.p217-224**

## INTRODUCTION

For the past three decades, there has been a dramatic increase in cases of recurring infections caused by multi-drug-resistant bacteria which is posing a threat to global public health.[1-2] Although there are many factors responsible for increasing antibacterial resistance, it is imperative to look for new materials and avenues as antimicrobial agents to manage the problem.[3] As the microorganisms gradually developed resistance to antibiotics, the need for an alternative to the prevailing antibiotics is obligatory. Calixarenes represents the well-known family of supramolecular compounds, which have been synthesized and evaluated for their ability to selectively recognize and remove different cation and anion molecules.[4] The reversible non-covalent interaction of calixarene with different analytes enhances its potential role in a variety of applications. The calixarene is a versatile molecular platform (Fig.1) that can be designed and developed into structural derivatives as new drugs.

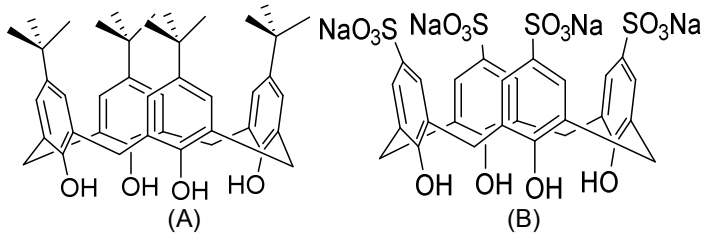


***Figure 1***
***(A) Basic structure of p-tertiary butyl calixarene;***
***(B) Sodium salt of p-sulfonato-calix-4-arene***

The characteristic property of calixarene as molecular platform for drug synthesis are: i) variable conformation, ii) cavity of appropriate size and architecture for ions and small molecules inclusion, iii) capability to recognize, bind and forms complexes with larger molecules, iv) capability of creating ditopic ligands with binding sites at the upper and lower rim of the parental compound, v) capability of combining ligands for the formation of molecular sensors, vectors, and switches vi) biocompatibility. Many pharmacological properties are described for calixarenes such as antiviral (including HIV),[5-6] antibacterial,[7] antifungal,[8] and anticancer activities.[9-10] Calixarene possesses intramolecular lipophilic cavities formed by aromatic rings of the macrocyclic skeleton; hence, they are hydrophobic in nature.[11] The supramolecular strategy to design calixarene derivative tethers to the development of hydrophobic molecules with limited solubility in biological media which made them unsuitable for *in vitro* standard evaluation as antimicrobial agents. Acquisition of optimum aqueous solubility is advantageous to measure the biological activities. Thus, synthetic strategies for the development of water soluble calixarene analog are preferred and researchers are introducing hydrophilic functions at the upper and lower rim of calixarene scaffold to make it more water soluble. Lamartine et al.[8] synthesized a series of water-soluble calixarenes derivatives wherein derivatives of p-sulfonato calixarene (SC4) have shown interesting biological activity with comparable growth inhibition against some bacterial species. Garrae et al[12] studied the *in vitro* activity of p-guanidinoethyl calix[4]arene and compared the results with hexamidine and chlorhexidine, two older cationic antiseptics. It has been established that p-guanidinoethyl calix[4]arene can present a good alternative to these two older antiseptics, which are characterized by higher cytotoxicity. Recently, oxygen bridged highly functionalized novel calix[2]arene[2]triazine derivatives[13] were evaluated for their effective antibacterial activity using docking studies. Hence, calixarene derivative such as sodium salt of p-sulfonato-calix-4-arene (calixarene derivative) with limited aqueous solubility could be a potential candidate as an antimicrobial agent. Over the past two decades alginate particles based therapeutics are being used for the treatment of various diseases, infection and inflammation as nano-carrier[14] with outstanding advantages including protection of the drug from premature degradation, the ability to deliver poorly-water soluble drugs alone or in combination with soluble drugs, controlled drug release mechanisms, improved biodistribution and pharmacokinetics and enhanced intracellular penetration with biodegradable and hydrophilic nature.[15-17] Alginate is a linear biopolymer obtained from brown algae. It contains β-d-mannuronate (M) and α-l-guluronate (G) residues linearly linked by 1,4-glycosidic linkages in varying proportions, sequences, and molecular weights.[18] Preparation of Ca-alginate nanoparticles (nanogels) for the development of nano-sized drug delivery systems has been reported.[19-20] In our laboratory we have prepared alginate nanogels using various divalent ions- ($Ca^{+2}$, $Ba^{+2}$ & $Sr^{+2}$) -as crosslinks and studied the effects of these crosslinks on the morphology, surface charge, protein encapsulation properties and stability of the resulting nanogels.[21] In the present study, we report the application of alginate nanogels as nano-carrier by encapsulation of sodium salt of p-sulfonato-calix-4-arene. The drug being hydrophobic with limited solubility in buffer requires a more hydrophilic carrier to reach the target cell efficiently. The usage of alginate nanogels commands the enhanced water solubility, controlled and sustained release rate. Alginate nanoparticles are important due to their size i.e., approximately in the range of 50-100 nm and hydrophilic character. The hydrogel nanoparticles were characterized with respect to their size, drug release profiles and its antimicrobial activity against drug-resistant strains of *E. coli* and compared with the antibacterial activity of sodium salt of p-sulfonato-calix-4-arene (calixarene derivative) partially soluble in the buffer.

# EXPERIMENTAL

## MATERIALS

Sodium alginate (mol. wt. 120,000–190,000) and hexane were purchased from Sigma-Aldrich. Polyoxyethylene sorbitan mono-oleate (Tween 80), calcium chloride, barium chloride, sodium chloride, sodium acetate, ethanol and glacial acetic acid were supplied by Fisher Scientific. Other chemicals such as peptone, beef extract, agar etc. were obtained from Qualigens India and Gentamicin disk (Hi-media, India), ethanol (AR). All chemicals were used as received. Deionized water was used throughout the experiments. *E.coli* (MTCC No.443) was used as a bacterial strain.

## METHODS

***Preparation of Calixarene derivative***

The parent p-tert-butyl calix[n]arene was prepared by condensing p-tert-butyl phenol with formaldehyde in the presence of sodium hydroxide as per the procedure reported earlier.[22-23] The sodium salt of a tetra-sulfonato derivative of calix[4]arene was also prepared through a procedure reported in the literature.[24] Expected spectroscopic data was obtained as per the literature report to confirm the formation of desired sodium salt of p-sulfonato-calix-4-arene.

***Preparation of alginate nanogels using $Ca^{+2}$/$Ba^{+2}$ as cross-linker and encapsulation of calixarene derivative***

Alginate solution was prepared in sodium acetate buffer saline by dissolving 0.2% sodium alginate in 100mM buffer solution (pH 7.4 at 4˚C) and incubated in orbital shaker for 2 hrs at 120 rpm to make a uniform alginate solution. 0.1M solution each of $CaCl_2$ and $BaCl_2$ was prepared for crosslinking with alginate. The sodium alginate solution was added dropwise to a vial containing organic phase (Hexane) and surfactant (Tween 80) under constant stirring for 30 min. and thereafter gelation was achieved by the addition of cation solution dropwise, which led to the crosslinking of the polymer chains and form Ba-alginate and Ca-alginate nanogel respectively. It is then washed with saline buffer under centrifuge to remove surfactant and hexane. Its hydrodynamic size was determined using dynamic light scattering (DLS). For the encapsulation of calixarene derivative into the alginate nanogel, the procedure remains the same except for premixing of calixarene derivative (0.243 mg/ml) with the 0.2% alginate solution.[25]

***Characterisation of calixarene derivative loaded alginate nanogel***

The size (hydrodynamic diameter) of calixarene derivative loaded alginate nanogels were measured using dynamic light scattering spectroscopy (DLS, a Malvern Zetasizer - Nano ZS, Malvern, UK).

***Solubility of calixarene derivative in PBS***

The water solubility of calixarene derivative was determined by dissolving the solid compound in 1 ml of water. For the study of biological activity, the calixarene derivative solution was prepared by gradually dissolving the above-mentioned amount of calixarene derivative in 1 ml of PBS (100mM; pH 7.4) and 1% ethanol. The mixture was vortexed in cold conditions to obtain a homogeneous suspension.[26]

***Absorption spectrum of calixarene in aqueous media***

Different concentrations of the aqueous solution of calixarene derivative (mg/ml) were used for spectrophotometric measurement from 200 nm to 800 nm and characteristic spectrum was obtained. A calibration curve was plotted by taking absorbance at 225 nm ($\lambda_{max}$) with respect to the concentration of aqueous calixarene derivative. It is further used to determine the unknown concentraton of calixarene derivative in samples.[27]

***Measurement of drug encapsulation efficiency and release kinetics of calixarene derivative from $Ca^{+2}$/ $Ba^{+2}$ alginate nanogel***

Calixarene derivative content in the alginate nanogel was determined by the method of de-crosslinking of alginate nanogels in brine solution given by Waldman et al.[28] In this method the breaking of cation-alginate crosslinks releases calixarene derivative, which was estimated spectrophotometrically by using the above-mentioned calibration curve (Section 2.4). Similarly, the residual calixarene derivative content of the wash solution was also determined spectrophotometrically. The drug encapsulation efficiency (EE) of the nanogels was calculated using the following equations:

$$EE = \frac{(A - B)}{A} \times 100\% \qquad (1)$$

Where A is the total amount of drug (calixarene derivative) and B is the residual calixarene derivative content. In a double-jacketed glass cell, at a constant temperature, 15ml of PBS buffer (pH 7.4) was taken as release media and 1ml calixarene derivative encapsulate in alginate nanogels was added as a dose. The release media was stirred at 120 rpm using digital magnetic stirrer. The calixarene derivative release was assessed by a sampling of release media at different time intervals up to 8 hrs and measuring the calixarene concentration at 225 nm spectrophotometrically. The experiment was repeated 3 times. A graph was plotted between the optical density (O.D.) and the corresponding time of sample collection (Fig.4) to evaluate the release kinetics of calixarene derivative.

***Biological Activity***

The biological activity of calcium and barium alginate nanogels loaded with the calixarene derivative (drug) was studied by viable count method, disc diffusion method and *E.coli* (MTCC 443) was used as test strain.[29-31]

***Antibacterial activity***

Antimicrobial activity of the test compound was carried out *in vitro* by using modified Kirby-Bauer disc diffusion method.[29,30] *E. coli* culture was inoculated into 5 ml of Mueller-Hinton Broth (MHB), and grown overnight for 18 h at 37°C with shaking at 120 rpm. 100 µl of the overnight grown cultures were inoculated into 100 ml MHB and incubated for 4 hrs at 37°C with shaking at 120 rpm. The 0.1ml culture was poured and carefully spread over the surface of solidified nutrient agar petri-plates. Sterile 6mm diameter discs impregnated with a different concentration of calixarene derivative solution were placed on the agar. The petri-plates were incubated at 37 °C for 12-16 hrs. Clear inhibition zones were then measured in millimeters (mm). Sterile water and gentamicin were taken as negative and positive control respectively. PBS (100mM; pH 7.4) was taken as a solvent control. Similarly, various concentration of nanogels (calcium and barium alginate) loaded calixarene derivative solution were made. Sterile 6mm diameter discs were impregnated with 5 µL of each prepared concentration. The discs were placed onto bacterial plates seeded with *E.coli* and were incubated at 37 ºC for 24 hrs, 48 hrs and 72 hrs, respectively. Simultaneously, negative control discs were prepared using the same solvent employed to dissolve the test compound and incubated as above. After the incubation period, the antimicrobial activity was examined by measuring the diameter of inhibition zone in mm. Tests were performed in triplicate.

***Cell viability test***

100 µl of the overnight grown *E. coli* were added to 100ml nutrient broth distributed in different flasks. Varying concentrations of the sample (Calixarene derivative) was added to each flask and incubated for 16 hrs at 37°C with shaking at 120 rpm. Similarly, various concentration of nanogels (calcium and barium alginate) loaded with calixarene derivative were added into separate flasks. Serial dilutions were made and 0.1 ml aliquot of each dilution was plated on solid nutrient agar plates followed by an overnight incubation at 37°C. The number of viable *E. coli* cells remaining in the suspension was measured by determining CFU/ml. The colonies formed in the agar gel were counted and colony forming units (CFU) per ml was calculated. Flask with only *E.coli* culture was taken as positive control. Only alginate nanogels crosslinked with $Ca^{+2}$/ $Ba^{+2}$ containing no calixarene derivative were also tested as a negative control.[31]

## RESULTS AND DISCUSSIONS

***Aqueous solubility of calixarene derivative***

The sodium salt of p-sulfonato-calix-4-arene (calixarene derivative) was synthesized as reported.[24] It contains sulfonato function at the upper rim as shown in Fig. 1B, which adds hydrophilic characters in the molecule or group while the presence of the 4-benzene ring in the center of the molecules renders it hydrophobic. Thus it has limited solubility in water as well as phosphate buffer saline (PBS) at pH 7.4 which reduces its biological application. We determined the aqueous solubility of the molecule by dissolving it in deionized water. The maximum solubility of the present molecule was found to be about 2.43 mg/ml of water. It was further dissolved in PBS for the evaluation of antimicrobial activity against *E. coli.* Figure 2 shows the absorption spectra of an aqueous solution of calixarene derivative with the characteristic absorption band at 225 nm. For the quantitative analysis of calixarene derivative, a series of the solution of known concentrations were measured at 225 nm, using a UV-Visible spectrophotometer. It gives the relationship between the absorbance and concentration of calixarene derivative. Thus the concentration of free calixarene derivative in aqueous solution was calculated using such a calibration graph.

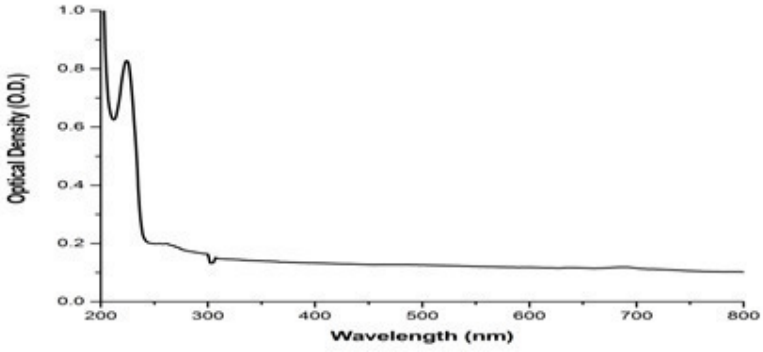


**Figure 2**

***Absorption spectrum of aqueous calixarene derivative showing absorption peak ($\lambda_{max}$) at 225nm.***

***Drug encapsulation and release kinetics from alginate nanogels***

The hydrophilic alginate nanogels were prepared by emulsion technique using divalent cations as the cross-links. Only $Ca^{+2}$ and $Ba^{+2}$ ions were used as the cross-links since they form stable nanogels as reported[21] and can be used for the high drug loading. The hydrodynamic diameter of calcium-alginate and barium alginate nanogels loaded with calixarene derivative (encapsulated calixarene derivative) was measured using dynamic light scattering experiments. It was found that the size of calcium alginate nanogels is smaller than barium alginate nanogels as shown in Figure 3. This may be caused by the larger size of $Ba^{+2}$ cation which could affect the coordination bond length in the alginate matrix.

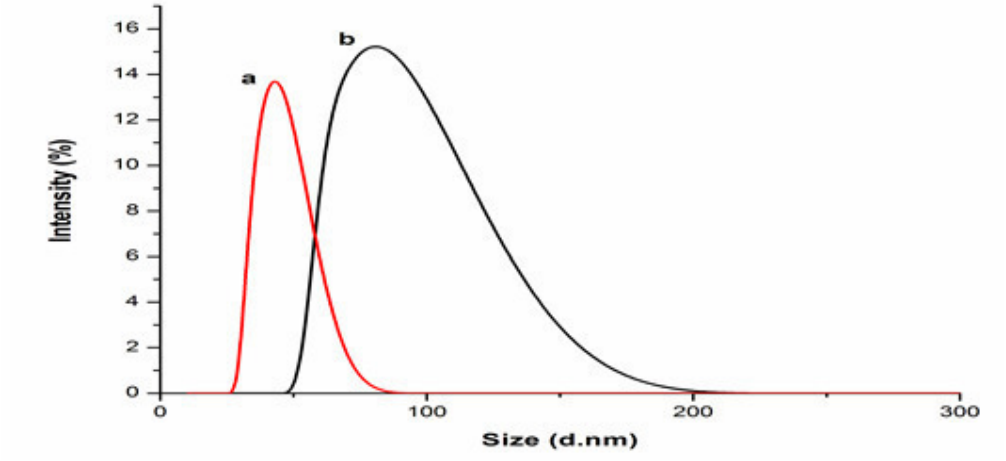


**Figure 3**
***Hydrodynamic diameter of alginate nanogels:***
***a). Calcium alginate nanogel, b). Barium alginate nanogel.***

The drug encapsulation efficiency of alginate nanogels was calculated as shown in equation (1) and it was found to be 90.4% and 87.2 % for Ca-alginate nanogel and Ba-alginate nanogel respectively. The drug release studies were performed in simulated serum fluids in double jacketed transport cell at 37°C. The diffusion rate of the sodium salt of p-sulfonato-calix-4-arene from alginate nanogels crosslinked with $Ca^{+2}$ and $Ba^{+2}$ was determined by measuring the concentration of calixarene derivative in simulated fluid spectrophotometrically as shown in Fig. 4. As expected, the rate of release is higher initially due to the presence of higher concentration of drug (calixarene derivative) in the nanogel matrix and behavior is diffusion dependent. The alginate matrix has no effect on initial diffusion of calixarene derivative from the alginate nanogel. The Ba-alginate nanogels have a slower rate of diffusion as compared to the calcium alginate nanogels. The release profile at physiological pH of 7.4 was significantly faster initially, and after about 6 hrs, the release of calixarene derivative was slow and sustained release profile was achieved. Alginate nanogels appear to be promising platform for therapeutics.

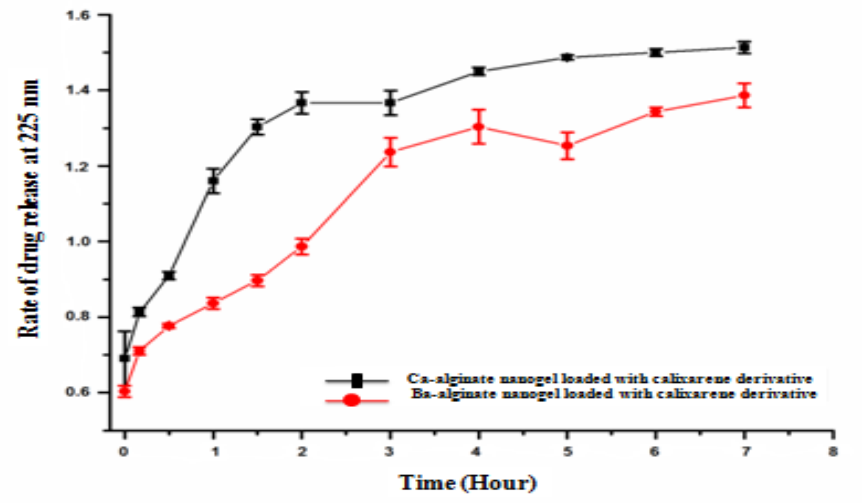


**Figure 4**
***The release profile of calixarene derivative encapsulated in different nano-alginate in phosphate buffer – saline***

***Microbial inhibitory activity of Calixarene derivative***

To study the microbial inhibitory activity of calixarene derivative, disk diffusion tests were performed.[29] Different concentration of calixarene derivative dissolved in buffer solution and encapsulated in alginate nanogels with different crosslinks was tested. Zone of inhibition was measured after 24 hrs and followed for 3 days. The significant antibacterial activity of calixarene derivative was observed after 24 hrs at the calixarene derivative concentration of 130 µg dissolved in a buffer as shown in Table 1. The alginate nanogel encapsulated calixarene derivative showed significant inhibitory effects after 24 hrs with the zone of inhibition of about 10 mm at the calixarene derivative concentration of 24 µg. In the case of alginate nanogels cross-linked with divalent cation containing no calixarene derivative, no measurable inhibition zone was observed towards microbes. Disc diffusion experiment is completely dependent on diffusion of a drug molecule in the solid agar. The limited solubility of calixarene derivative in

PBS solution restricts the availability of drug molecules for action and in turn shows the lower impact of calixarene derivative on microbial culture. The encapsulation of calixarene derivative in hydrophilic nanogels may increase the accessibility of calixarene derivative to the *E.coli* culture which subsequently showed a microbial inhibitory effect at a much lower concentration of drug molecule i.e. calixarene derivative.

**Table 1**
**Measurement of Zone of Inhibition (mm), obtained by the disc diffusion test for aqueous calixarene derivative and encapsulated calixarene derivative in calcium alginate nanogels and barium alginate nanogel.**

| S.No. | Sample | Diameter of Zone of Inhibition (mm) | | |
|---|---|---|---|---|
| | | 24 hrs | 48 hrs | 72 hrs |
| 1. | Aqueous calixarene derivative (130µg) | 8±0.30 | 10±1.04 | 10±0.61 |
| 2. | Calcium alginate nanogels | 6±0.49 | 6±0.66 | 6±0.50 |
| 3. | Encapsulated calixarene derivative in calcium alginate nanogels (24µg) | 10±1.00 | 12±0.85 | 20±1.15 |
| 4. | Barium alginate nanogels | 6±0.47 | 6±0.40 | 6±0.25 |
| 5. | Encapsulated calixarene derivative in barium alginate nanogels (22µg) | 8±0.76 | 8±1.37 | 9.5±1.5 |
| 6. | Gentamicin (10µg) | 22±1.79 | 22±0.40 | 22±1.15 |

***[*Note: Calcium alginate nanogels = Calcium alginate nanogels with no calixarene derivative; Barium alginate nanogel = Barium alginate nanogels with no calixarene derivative.] ± SD, n=6***

***Effect of aqueous calixarene derivative and encapsulated calixarene derivative on Microbial cell viability***

The untreated bacterial sample showed $3x10^6$ CFU/ml after 24 hrs which were taken as reference to measure the CFU value for the *E.coli* samples treated with aqueous calixarene derivative and encapsulated calixarene derivative. The inhibitory effect was observed at a concentration of 2.43 mg of aqueous calixarene derivative with CFU/ml of $1.20x10^6$, $0.82x10^6$,$0.52x10^6$at 24 hrs, 48hrs, and 72 hrs respectively, as shown in Table 2. These were significantly lower than CFU/ml values observed for control experiments. In the case of encapsulated calixarene derivative in the $Ca^{+2}$/$Ba^{+2}$ alginate nanogels, the decrease in CFU/ml were also observed after 24 hrs at a lower concentration of calixarene derivative (24.3µg and 12.15µg). Reduction in the colony count is seen from 24 hrs onwards which could be due to the enhancement of hydrophilicity of calixarene derivative encapsulated in alginate nanogels. Similar results were obtained with calixarene derivative encapsulated in barium alginate. In comparison to the free calixarene derivative in aqueous solution, the encapsulated calixarene derivative has a larger inhibitory effect on *E. coli* cell viability at a lower concentration.

**Table 2**

***Effect of calixarene derivative on E.coli cell viability***

| S.No. | Sample | Concentration of Calixarene derivative | Day 1 (106 CFU/ml) | Day 2 (106 CFU/ml) | Day 3 (106 CFU/ml) |
|---|---|---|---|---|---|
| 1. | Positive control (*E.coli* culture) | Nil | 3.0±0.2 | 3.45±0.21 | 3.8±0.35 |
| 2. | Aqueous calixarene derivative (500 µl) | 1.215 mg | 1.2±0.37 | 0.8±0.06 | 0.57±0.1 |
| 3. | Aqueous calixarene derivative (1000 µl) | 2.43 mg | 0.99±0.09 | 0.8±0.43 | 0.54±0.10 |
| 4. | Ca-alginate nanogels (100 µl) | Nil | 2.94±0.04 | 2.92±0.06 | 2.84±0.11 |
| 5. | Calixarene derivative encapsulated in Ca-alginate nanogels (500 µl) | 10.98 µg | 0.71±0.14 | 0.62±0.09 | 0.58±0.05 |
| 6. | Calixarene derivative encapsulated in Ba-alginate nanogels (1000 µl) | 21.96 µg | 0.6±0.08 | 0.55±0.01 | 0.39±0.02 |
| 7. | Ba-alginate nanogels (100 µl) | Nil | 2.74±0.07 | 2.61±0.11 | 2.52±0.21 |
| 8. | Calixarene derivative encapsulated in Ba-alginate nanogels (500 µl) | 10.59 µg | 0.84±0.43 | 0.79±0.07 | 0.75±0.04 |
| 9. | Calixarene derivative encapsulated in Ba-alginate nanogels (1000 µl) | 21.18 µg | 0.62±0.1 | 0.52±0.05 | 0.51±0.04 |

**± SD, n=6**

## CONCLUSION

In the present study, calixarene derivative with limited aqueous solubility has been successfully encapsulated in hydrophilic alginate nanogel cross-linked with $Ca^{+2}$ / $Ba^{+2}$ ions and the corresponding nanogels exhibit stable size of 60 nm and 110 nm respectively. The effect of various concentration of calixarene derivative on *E.coli* growth was efficaciously studied by disc diffusion method and CFU method. Antimicrobial activity of calixarene derivative tested by disc diffusion method at various concentration of aqueous calixarene derivative could effectively demonstrate zone of inhibition at 130 µg concentration of calixarene derivative. In the case of encapsulated calixarene derivative in $Ca^{+2}$ and $Ba^{+2}$ alginate nanogels, the zone of inhibition appeared at the concentration of 24 µg and 22 µg respectively, of calixarene derivative which further increases till the third day. Encapsulated calixarene derivative in barium alginate nanogels also showed a significant increase in the zone of inhibition at 22 µg of encapsulated calixarene derivative but the diameter of the zone of inhibition was smaller in comparison to encapsulated calixarene derivative in calcium alginate nanogels. The superior performance of calixarene derivative

encapsulated in $Ca^{+2}/Ba^{+2}$ alginate nanogels suggest that hydrophilic alginate nanogels could be a new nano platform for the delivery of therapeutics like calixarene derivative having limited solubility in aqueous systems. The sustained release of encapsulated calixarene derivative may impact the constant interaction of calixarene derivative molecule and the microbe in the aqueous environment which might affect the efficacy of microbial activity. Further investigation on the nano platform is underway to find niche performance towards better clinical translation.

## ACKNOWLEDGEMENT

This work was supported by the Department of Biotechnology, New Delhi, India under a Bio-CARe women scientist scheme award to RS, [Grant No. BT/Bio-CARe/05/640/2011-12].

## CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript